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(54) Title: ACID-LABILE AND ENZYMATICALLY DIVISIBLE DYE COMPOUNDS FOR DIAGNOSIS WITH NEAR INFRARED LIGHT AND FOR THERAPY


(57) Abstract

The invention relates to acid-labile and enzymatically divisible compounds for in-vitro and in-vivo diagnosis by means of near infrared radiation (NIR radiation), the use of said compounds as optic diagnostic and therapeutic agents, and the diagnostic agents containing said compounds.

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"Acid-Labile and Enzymatically Divisible Dye Systems for Diagnosis with Near-Infrared Light and for Therapy"

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## Description

This invention relates to acid-labile and enzymatically divisible compounds for in-vivo and in-vitro diagnoses employing near-infrared (NIR) radiation, the use of such compounds for diagnostic and therapeutic purposes, and substances containing these compounds.

Near-infrared imaging is a noninvasive diagnostic process which takes advantage of the high level of transmissivity of biological tissue to light in the 650-1000 nm wavelength range. In contrast to light in the ultraviolet and visible spectral regions which can penetrate only the outermost few millimeters of the tissue, near-infrared light can penetrate tissue as deep as several centimeters. The reason for the basically minor penetrating depth of light lies in its absorption by pigments in the body, chiefly in hemoglobins and water, a phenomenon of insignificant proportions, however, in the near-infrared spectral range between 650 and 1000 nm. This spectral region of maximum optical tissue transparency has therefore been referred to as a diagnostic/therapeutic window (Boulnois, J., Lasers Med Sci 1986, 1:47-66). The diagnostician can thus avail himself of another tissue-imaging tool in addition to such modern imaging processes as radiography, magnetic resonance tomography or ultrasonography (Haller, E.B., "Time-resolved Transillumination and Optical Tomography", J Biomed Optics 1996, 1:7-17).

Procedures employing NIR radiation for the localized detection of blood flow and the degree of oxygenation in the brain of infants by quantizing hemoglobin/deoxyhemoglobin absorption have been known and applied for years (Jöbsis, F.F., Science 1977, 198:1264-67; Chance, B., Leigh, J.S., Miyake, H. et al, Proc Natl Acad Sci USA 1988, 85:4971-75; Benaron, D.A. et al, Science 1993, 33:369A.).

The main problem in the use of near-infrared radiation is the considerable scattering of the light whereby even a narrowly delineated object with photophysical properties different from the surrounding area is only poorly contrasted. The farther the object from the surface, the greater the image-quality problem, which can be considered to be the principal limiting factor both in transillumination and in the detection of fluorescence radiation. This is why dyes, used as contrast agents which intensify the optical properties of the tissue as well as the absorption and fluorescence of the tissues to be examined, permit unambiguous detection even if spatial resolution is limited. In the process, the absorptivity of the dye compounds can serve to provide the graphic information. If in addition the dyes have the ability to re-emit the energy absorbed in the form of fluorescent radiation, the latter as well can serve to provide pictorial data. In this case the fluorescent radiation, red-shifted relative to the excitation radiation, is separately imaged. One of the advantages in this is that in the NIR range the tissue itself displays very little intrinsic fluorescence of its own which minimizes background noise.

(Folli, S. et al, Cancer Research 54, 2643-9 (1994); Ballou, B. et al, Cancer Immunol. Immunother. 41, 257-63 (1995); Li, X. et al, SPIE Vol. 2389, 789-98 (1995)).

In fluorescence-based diagnoses it is necessary to have sufficient, maximum possible differentiation between the fluorescence emitted by the object tissue under analysis and that of the surrounding tissue. This is typically obtainable by virtue of a change in the concentration of the fluorescent dye at a certain time following the application of the substance. For diagnoses in lower-lying tissue layers, this differential change is often insufficient when substances with nonspecific contrast-enhancing patterns are used.

This invention is therefore aimed at providing new compounds which remedy these prior-art shortcomings.

According to the invention, this is accomplished using compounds of the general formula (I)

$$(F-L)_m-A \qquad (I)$$

where

F is a dye molecule having at least one maximum absorption peak between 600 and 1200 nm,

L is a linker structure containing an acid-labile and/or enzymatically divisible link,

m is a number between 1 and 80,

in which case, if m is a number between 1 and 3,

A represents a dye molecule having at least one maximum absorption peak between 600 and 1200 nm, an antibiotically or anticytostatically effective molecule, a biomolecule, a nonbiological macromolecule or a $B-(L-W)_o$ or $D-(L-W)_o$ compound in which

D is a nonbiological macromolecule,
B is a biomolecule,
L is as stated above,
W is an antibiotically or anticytostatically effective molecule,
o is a number between 1 and 20,

and if m is a number between 4 and 80,

A represents a biomolecule, a nonbiological macromolecule or a $B-(L-W)_o$ or $D-(L-W)_o$ compound in which

D, B, L, W and o signify the same as above.

The outstanding feature of the compounds according to this invention relative to in-vivo detection of near-infrared fluorescence emission is their total or near-absence of intrinsic fluorescence emission, and that it is only after a division of the system i.e. the splitting-off of the dye from the system at the target (e.g. a tumor or infection) that the fluorescence signal is augmented. Accordingly, the effective difference of the fluorescence signal between the object tissue under analysis and the surrounding tissue is established by virtue of

a) the concentration differential due to pharmacokinetic mechanisms and

b) the difference in the quantitative fluorescence yield at the time of the diagnosis.

It has been found that the fluorescence of dyes is quenched when in producing the compounds according to this invention a dye molecule is conjugated with another molecule (dimer), meaning that there is extremely little fluorescence emission compared to a corresponding dye molecule in the unconjugated state. It has also been found that comparable quenching is obtained when other molecules having aromatic structures, be they dyes or active agents (e.g. cytostatics or antibiotics), are conjugated with the fluorescent dye. And, surprisingly, quenching also occurs when the dyes are conjugated with antibodies, antibody fragments and proteins.

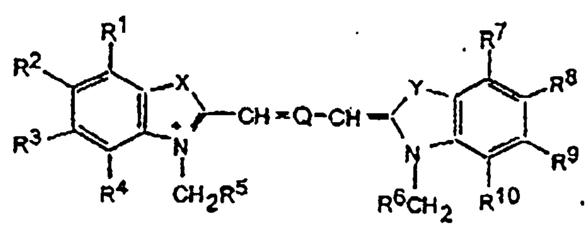
As a fundamental requirement, the dyes which are a structural component of the compounds according to this invention must offer high molecular coefficients of absorption and a high quantitative fluorescence yield in their unconjugated, monomer form.

In preferred compounds of the general Formula I according to this invention, F and/or A characteristically represent a

polymethine dye, tetrapyrrole dye, tetraazapyrrole dye, xanthine dye, phenoxazine dye or phenodiazine dye.

Particular preference is given to structures of the polymethine dye category in view of their absorption maxima with very high molecular absorption coefficients in the near-infrared spectral region between 700 and 1000 nm ($\varepsilon$ up to 300,000 l $mol^{-1}$ $cm^{-1}$), for instance cyanine dyes, squarilium dyes and croconium dyes as well as merocyanine and oxonol dyes.

Also preferred are those compounds of the general Formula (I) according to this invention in which F and/or A represents a cyanine dye of the general Formula II




(II)

where

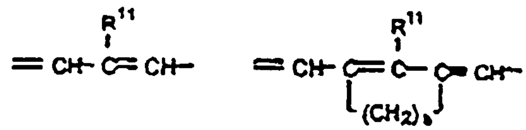
$R^1$ to $R^4$ and $R^7$ to $R^{10}$ independently of one another represent a fluoro-, chloro-, bromo-, iodo-atom or a nitro group or a residual $-COOE^1$, $-CONE^1E^2$, $-NHCOE^1$, $-NHCONHE^1$, $-NE^1E^2$, $-OE^1$, $-OSO_3E^1$, $-SO_3E^1$, $-SO_2NHE^1$, $E^1$,

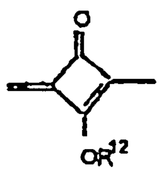
where $E^1$ and $E^2$ independently of one another represent a hydrogen atom, a saturated or unsaturated, branched or linear $C_1$-$C_{50}$-alkyl chain, which chain, or parts thereof, may form one or several aromatic or saturated cyclic $C_5$-$C_6$ or bicyclic $C_{10}$ units while the $C_1$-$C_{50}$ alkyl chain is interrupted by 0 to 15 oxygen atoms and/or 0 to 3 carbonylic groups and/or is substituted by 0 to 5 hydroxy groups, 0 to 5 ester groups, 0 to 3 carbon groups, 0 to 3 amino groups,

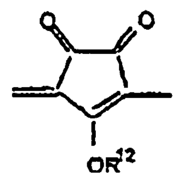
and where respectively juxtapositioned residuals of $R_1$ - $R_4$ and/or $R_7$ - $R_{10}$ may be linked with one another under formation of a six-link aromatic carbon ring, $R^5$ and $R^6$ independently of each other represent a residual $-E^1$ as identified above or a $C_1$-$C_4$ sulfoalkyl chain,

and/or $R^1$ to $R^{10}$ represent a link with L,

Q is a fragment,







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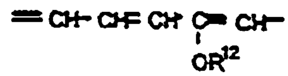
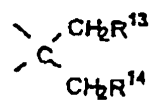


where $R^{11}$ represents a hydrogen, fluoro-, chloro-, bromo-, iodo-atom or a nitro group or a residual $-NE^1E^2$, $-OE^1$ or $-E^1$, with $E^1$ and $E^2$ identified as above, or a link with L,

$R^{12}$ represents a hydrogen atom or a residual $E^1$ as identified above,

b is a number 0, 2 or 3,

X and Y independently of each other represent O, S, -CH=CH- or a fragment



where

$R^{13}$ and $R^{14}$ independently of eachother represent hydrogen, as a saturated or unsaturated, branched or linear $C_1$ - $C_{10}$ alkyl chain which may be interrupted by up to 5 oxygen atoms and/or substituted by up to 5

hydroxy groups, and where the residual $R^{13}$ and $R^{14}$ may be mutually linked under formation of a 5- or 6-link ring.

Another object of this invention includes compounds of the general Formula (I) in which dyes are linked with a therapeutically effective molecule by way of a physiologically breakable bond, or the dye and active agent are conjugated via physiologically divisible bonds with biomolecules or nonbiological carrier molecules.

Particular preference is given to systems in which the fluorescence of the dye in the conjugated state is quenched and the therapeutic activity of the active molecule is masked by the conjugation with the dye or carrier molecule (prodrug effect). The breaking of the bond leads to an augmentation of the fluorescence emission while at the same time releasing the activity of the active agent.

The active agents W and/or A in the general Formula (I) according to this invention consist, for example, in the following compounds:

Antibiotics: Aclacinomycin, actinomycin $F_1$, anthramycin, azaserine, bleomycin, cactinomycin, carubicin, carcinophilin, chromomycin, dactinomycin, daunorubicin, doxorubicin, epirubicin, mtiomycin, mycophenolic acid, nogalamycin, olivomycin, peplomycin, plicamycin, porfiromycin, puromycin, streptonigrin, tubercidin, zorubicin;

Folic acid analogues: Denopterin, metothrexate, pteropterin, trimetrexate;

Pyrimidine analogues: Ancitabin, azacitidine, 6-azauridine, carmofur, cytarabine, doxifluridine, enocitabine, floxuridine, 5-fluorouracil;
Purine analogues: Fludarabine, 6-mercaptopurine, thiamiprine, thioguanine and derivatives of these compounds;
Alkylating substances: Alkyl sulfonates, aziridines, ethylenimines, methyl melamines, nitroureas, nitrolostic compounds;
Hormonally active substances such as androgens, antiadrenals, antiandrogens, antiestrogens, estrogens;
LH-RM analogues and progestogens;
as well as other cytostatically effective substances such as taxol and taxol derivatives.

Other effective agents include photodynamically active substances which have the ability, upon excitation, to develop a photosensitizing effect through the formation of cytotoxic singlet oxygen and of radicals. Compounds of this type include primarily tetrapyrroles or tetraazapyrroles, for example porphyrines, benziporphyrines, chlorines, purpurines, pthalocyanines, naphthaocyanines and derivatives of these compounds. Other compounds include expanded porphyrines, porphycenes and oxazine or phenoxazine dyes.

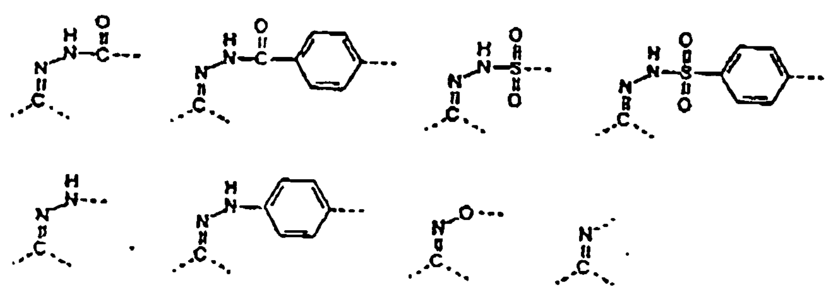
The chemical bond contained in the link structure L according to the general Formula (I) is structured in a manner that certain physiological parameters characterizing deseased tissue (tumors) and distinguishing themselves from normal tissue areas will split i.e. break that bond.

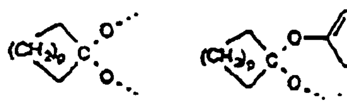
Existing literature on the subject states that tumors characteristically have a lower pH than normal tissue. Whereas the intracellular pH value is largely

identical (approx. pH 7.4), the extracellular pH in tumors is up to 0.5 pH units lower. Infections as well, especially those bacterial in nature, display lower pH values. The methods for determining the pH values include, inter alia, measurements with microelectrodes, fluorescence measurements using pH-sensitive fluorescence probes, and measurements with MR probes.

(R.J. Gillies et al, Am. J.Physiol. 267, pC 195-203 (1994);
G.R. Martin and R.K. Jain, Microvascular Research 46, 216-230 (1993);
L.E. Gerweck and K. Seetharaman, Cancer Research 56, 1194-1198 (1996);
K. Engin et al, Int. J. Hyperthermia 11 (1995) 211-216;
K. Engin et al, Int. J. Radiation Oncology Biol. Phys. 29 (1994) 125-132;
G. Helmlinger et al, Nature Medicine 3 (1997) 177-182.

Another object of this invention therefore includes compounds with link structures L which are divided by reduced physiological pH values. Examples of structures of this nature include alkyl hydrazones, acyl hydrazones, aryl hydrazones, sulfonyl hydrazones, imines, oximes, acetals, ketals, orthoesters corresponding to these fragments:



 or 

where p represents a number between 2 and 4.

Apart from a dissociation due to reduced pH values, the splitting of the compounds according to this invention can also occur as a result of enzymes which are present at increased concentrations in the tissues under analysis (such as tumors, bacterial infections).

Another object of this invention therefore includes compounds with link structures L which are divided enzymatically. Examples of enzymatically dissociable link structures include those which are split by cathepsins, peptidases, carboxylic peptidases, α- and ß-glucosidases, lipases, oxidases, phospholipases, phosphatases, phosphodiesterases, proteases, elastases, sulfatases, reductases, transferases and bacterial enzymes such as penicillin amidases as well as ß-lactamases (P.D. Senter et al, Bioconjugate Chem. 6 (1995), 389-94).

Preferred, enzymatically dissociable structures include short-chain peptide sequences, for instance those which contain the Val-Leu-Lys amino acid sequence.

The kinetics leading to an enrichment in the tissue under analysis, i.e. to a corresponding concentration gradient at a specific time after the application,

must correlate with the kinetics of the division of the compounds according to this invention and with the kinetics of the evacuation of the released dye molecule, thus producing a synergistic effect.

Other preferred compounds according to this invention along the general Formula (I) are characterized by the fact that A and/or B represents an antibody, its conjugates and fragments, specific peptides and proteins, receptors, enzymes, enzyme substrates, nucleotides, natural or synthetic ribonucleic acids or desoxyribonucleic acids or their chemical modifications such as aptamers or antisenseoligonucleotides, lipoproteins, lectines, carbohydrates, mono-, di- or trisaccharides, linear or branched oligo- or polysaccharides or saccharide derivatives or a dextran.

Also preferred are the compounds according to this invention along the general Formula (I) in which D represents polyethylene glycol, polypropylene glycol, polylysin or polylysin dendrimers or their derivatives.

The linking of the structural elements A, D, B, L and W takes place either directly or by way of typical functional groups. Examples of such groups include ester, ether, secondary and tertiary amines, amides, thiourea groups, urea groups, carbamate groups or maleimide structures.

Another object of this invention is the use of the compounds according to the invention along the general Formula (I) for the in-vivo diagnosis of deseased tissue areas employing NIR radiation as well as for the therapeutic treatment of diseased tissue areas.

Another object of this invention is an optical diagnostic agent for the in-vivo diagnosis of deseased tissue areas employing NIR radiation which agent

contains at least one compound according to this invention along the general Formula (I).

The compounds are produced employing conventional methodology, with the use, if necessary, of traditional additives and/or carrier substances, diluents and the like. These include physiologically compatible electrolytes, buffers, detergents and substances for adapting the osmolarity and enhancing stability and solubility. Sterility of the preparations in the production process and especially prior to application must be assured by the traditional provisions and measures of the pharmaceutical industry.

The synthesis of the F and A dyes is performed by methods described in earlier literature such as:

F.M. Hamer, "The Cyanine Dyes and Related Compounds", John Wiley and Sons, New York, 1964;

J. Fabian et al, Chem. Rev. 92 (1992) 1197;

L.A. Ernst et al, Cytometry 10 (1989) 3-10;

P.L. Southwick et al, Cytrometry 11 (1990) 418-430;

R.B. Mujumdar et al, Bioconjugate Chem. 4 (1993) 105-11;

E. Terpetschnig et al, Anal. Biochem. 217 (1994) 197-204;

J.S. Lindsey et al, Tetrahedron 45 (1989) 4845-66;

EP-0591820 A1;

L. Strekowski et al, J. Heterocycl. Chem 33 (1996) 1685-1688;

S.R. Mujumdar et al, Bioconjugate Chem. 7 (1996) 356-362;

M. Lipowska et al, Synth. Commun. 23 (1993) 3087-94;

E. Terpetschnig et al, Anal. Chim. Acta 282 (1993) 633-641

M. Matsuoka and T. Kitao, Dyes Pigm. 10 (1988) 13-22; and

N. Narayanan and G. Patronay, I. Org. Chem. 60 (1995) 2361-95.

Employing methods described in earlier literature, the dyes are synthesized with substituents containing acid-labile or enzymatically divisible bonds or from which such bonds are produced upon conjugation, for instance as described in the following:

B.M. Mueller et al, Bioconjugate Chem 1 (1990) 325-330;

K. Srinivasachar and D.M. Neville, Biochemistry 28 (1989) 2501-09;

D.M. Neville et al, J. Biol. Chem. 264 (1989) 14653-61;

T. Kaneko et al, Bioconjugate Chem. 2 (1991), 133-41;

B.A. Froesch et al, Cancer Immunol. Immunother. 42 (1996) 55-63; and

J.V. Crivello et al, J. Polymer Sci., Part A: Polymer Chem 34 (1996) 3091-3102.

The following examples serve to explain this invention.

Examples:

1. <u>Synthesis of 5-(1-oxoethyl)-1,1'-(4-sulfobutyl)-indotricarbocyanine sodium salt 1</u> (fig. 1)

4-hydrazinophenyl methyl ketone is synthesized from 4-aminophenyl methyl ketone by diazotizing and reduction with $SnCl_2$ (based on the method by T. Górecki et al, J. Heterocyclic Chem. 33 (1996) 1871-76).
4.8 g (32 mmol) of 4-hydrazinophenyl methyl ketone, 5.4 g of sodium acetate and 3.9 g (45 mmol) of 3-methyl-2-butanone are agitated in 40 ml acetic acid for 1 hour at room temperature and for 4 hours at 120°C. The reaction mixture

is evaporated in a vacuum, introduced in 300 ml dichloromethane and the organic phase is rinsed with a saturated NaCl solution. After drying over $MgSo_4$ the resulting product is 7.5 g of a brown oil. This is heated with 6.5 g (48 mmol) of 1.4-butane sultone for 5 hours to 140°C, then cooled, mixed with acetone, and the precipitated solids are chromatographically purified (RP C-18, fluidizer methanol/water). Yield: 2.5 g (23%) of 5-(1-oxoethyl)-1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenine **2**.

To produce dye **1**, 0.5 g (1.7 mmol) of 1-(4-sulfobutyl)-2,3,3-trimethyl-3H-indolenine **3** is agitated with 0.47 g (1.6 mmol) of glutaconaldehyde dianil hydrochloride in 10 ml acetic anhydride for 30 minutes at 120°C. After cooling it is mixed with 0.6 g (1.8 mmol) of **2**, 10 ml acetic anhydride, 4 ml acetic acid and 0.5 g sodium acetate and heated for 30 minutes to 120°C. The deep-blue solution is cooled, stirred with 200 ml ether and the precipitated solids are filtered off. After chromatographic purification (RP C-18, fluidizer methanol/water) and freeze-drying, the result is 0.3 g (26%) of product **1**.

Elemental analysis:

| | | | | |
|---|---|---|---|---|
| Rge: | C 61.99 | H 6.33 | N 3.91 | S 8.95 |
| Grdt: | C 61.73 | H 6.49 | N 3.80 | S 8.78 |

Absorption: $\lambda_{max}$ ($H_2O$) = 748 nm ($\varepsilon$ = 148000 l $mol^{-1}$ $cm^{-1}$)

2. Modification with acid-labile linkers (fig. 2)

2.1 Modification of **1** with 4-carboxyphenyl sulfonyl hydrazine

0.2 g (0.28 mmol) of **1** and 74 mg (0.34 mmol) of 4-carboxyphenyl sulfonyl hydrazine are dissolved in 20 ml methanol, mixed with 5 µl trifluoroacetic acid agitated for 18 hours at room temperature. The solvent is evaporated in a

vacuum, the residue is rinsed several times with dichloromethane and the product is then dried. Yield: 0.21 g of **4**.

2.2 Modification of **1** with 4-aminobenzoic acid hydrazide

0.2 g (0.28 mmol) of **1** and 51 mg (0.34 mmol) of 4-aminobenzoic acid hydrazide are modified as per 2.1. Yield: 0.20 g of **5**.

2.3 Modification of **1** with 4-(aminomethyl)benzoic acid hydrazide

0.2 g (0.28 mmol) of **1** and 56 mg (0.34 mmol) of 4-aminomethyl benzoic acid hydrazide are modified as per 2.1. Yield: 0.22 g of **6**.

3. Producing reactive functional groups (N-hydroxysuccinimide ester and isothiocyanate) (fig. 2)

For producing the corresponding N-hydroxysuccinimidyl ester compound **7**, 0.1 g (0.1 mmol) of **4** is prepared with 14 mg (0.12 mmol) of N-hydroxysuccinimide (NHS) in 12 ml dimethyl formamide (DMF) and mixed at room temperature with a solution of 23 mg (0.11 mmol) of dicyclohexyl carbodiimide in 1 ml of DMF. After agitating for 72 hours the product is precipitated with diethyl ether, filtered out and again precipitated from DMF/diethyl ether. After vacuum drying the product obtained (12 mg) can be used without further purification.

For producing the acid-labile isothiocyanate compound **8**, 0.1 g (0.11 mmol) of **5**, 33 mg (0.14 mmol) of N,N'-thiocarbonyldi-2(1H)-pyridone and 15 mg (0.15 mmol) of triethylamine are agitated in 15 ml chloroform for 60 minutes at room temperature. The product is precipitated with diethyl ether, filtered out and

purified with MPLC (RP Select B, Merck, fluidizer 10mM phosphate buffer pH 8 / methanol). The result is 40 mg (40%) of **8** after freeze-drying, separation of the salts with dichloromethane/methanol and vacuum-drying.

4. Labeling of mAK 9.2.27 (anti-melanoma antibody)

4.1 Labeling with acid-labile NHS ester **7**.

1 mg of antibody in 0.5 ml 50 mM borate buffer (pH 9.2) is mixed with 33 µl of **7** (stock solution 5 mmol/l in DMF) and agitated for 1 hour at room temperature. Uncombined dye is separated via NAP-5 columns (elutriation with 25 mM phosphate buffer pH 7.8, +0.01% $NaN_3$). The resulting mAK9.2.27/**4** conjugate is stored in a solution at 4°C.

The VIS/NIR absorption spectrum of mAK9.2.27/4 conjugate (in a phosphate buffer pH 7.8) is shown in figure 5.

The quantitative fluorescence yield Q = 0.1% (5 µmol/l in a phosphate buffer

pH 7.8; as referenced against indocyanine green as the standard with Q = 13% in DMSO according to R.C. Benson and H.A. Kues, J. of Chemical and Engineering Data 22 (1977) 379).

4.2 Labeling with acid-labile isothiocyanate **8**

1 mg of antibody in 0.5 ml 50mM borate buffer (pH 9.2) is mixed with 6 µl of **8** (stock solution 5 mmol/l in DMF) and agitated for 15 minutes at room temperature. Uncombined dye is separated via NAP-5 columns (elutriation with 25 mM phosphate buffer pH 7.4, +0.01% $NaN_3$). The resulting mAK9.2.27/5 conjugate is stored in a solution at 4°C.

5. Synthesis of dimerous indotricarbocyanine dyes

5.1 Producing the symmetrical spirodimer **10** (fig. 3)

0.1 g (0.47 mmol) of 3.9-diethylidene-2,4,8,10-tetraoxaspiro-[5.5]undecane (synthesized per M. Crivello et al, J. Polymer Sci., Part A: Polymer Chem. 34 (1996) 3091-3102) and 0.11 g (0.94 mmol) of 6-amino-1-hexanol are agitated in 15 ml of diethyl ether for 24 hours at room temperature and the solvent is evaporated in a vacuum. The residue is dried on the oil pump and converted without further purification.

0.2 g (0.28 mmol) of 5-carboxy-bis-1,1'-(4-sulfobutyl)-indotricarbocyanine sodium salt **9** is agitated in 15 ml dichloromethane together with 0.09 g (0.28 mmol) of TBTU and 30 mg of triethylamine for 30 minutes and mixed with 0.06 g (0.14 mmol) of the above-mentioned spiro compound in 2 ml of dichloromethane. After 18 hours of agitation at room temperature the product is precipitated with diethyl ether and chromatographically purified (RP C-18, fluidizer methanol/10 mM phosphate buffer pH 8). After freeze-drying the salts are

precipitated with methanol/dichloromethane. The result is 68 mg (26%) of product **10**.

The VIS/NIR absorption spectrum of 10 (5 µmol/l in phosphate buffer pH 8) is shown in fig. 6.

The quantitative fluorescence yield Q = 0.2% (5 µmol/l in phosphate buffer pH 8, as referenced against indocyanine green as a standard, see example 4.1).

5.2 Producing a dye dimer (**11**) with an acid-labile hydrazone linker from **6** (fig. 4).

0.1 g (0.14 mmol) of 5-carboxy-bis-1,1'(4-sulfobutyl)-indotricarbocyanine sodium salt **9** is agitated in 10 ml DMF together with 45 mg (0.14 mmol) of TBTU and 15 mg of triethylamine for 30 minutes and mixed with 0.14 g (0.16 mmol) of **6** in 2 ml DMF. After 5 hours of agitation at room temperature the product is crystallized out by the admixture of diethyl ether, filtered out and chromatographically purified (RP C-18, fluidizer methanol / 10 mM phosphate buffer pH 8). After freeze-drying the salts are separated with methanol/ dichloromethane. The result is 0.13 g (59%) of **11**.

The VIS/NIR absorption spectrum of **11** (4 µmol/l) in a phosphate buffer pH 8.0 and in a phosphate buffer pH 6.0 after 24 hours at 37°C is shown in fig. 7.

6. <u>Measuring the quantitative fluorescence yield of **11** at different pH values as a function of time</u>

Solutions with concentrations of 4 µmol/l in 50 mM phosphate buffer having pH values of 7.4, 7.0, 6.6, 6.0 and 5.0 were incubated at 37°C. Aliquot samples were taken at different times for determination of the quantitative fluorescence yield (SPEX Fluorolog spectrofluorometer, 400 W Xe lamp, PM958 detector calibrated for wavelength-dependent detector response, all values referenced against indocyanine green -- see example 4.1).

Figure 8 shows the dissociation process of the acid-labile dimer **11** expressed by the progressive increase in the quantitative fluorescence yield at different pH values as a function of time.

7. Synthesis of a doxorubicin-indotricarbocyanine conjugate (13) with an acid-labile hydrazone linker (fig. 4)

20 mg (34 µmol) of doxorubicin-hydrochloride and 11 mg (68 µmol) of 4-(aminomethyl) benzoic acid hydrazide are agitated in 3 ml of anhydrous methanol, after the admixture of 2 µl of trifluoroacetic acid, for 24 hours at room temperature. The product **12** is extracted by crystallization with acetonitrile, separated by centrifuge, rinsed with acetonitrile, and dried. The raw-product yield is 18 mg (24 µmol). 14 mg (20 µmol of 5-carboxy-bis-1,1'-(4-sulfobutyl)-indotricarbocyanine sodium salt **9** is agitated in 0.5 ml of DMF together with 7 mg (22 µmol) of TBTU and 20 µl of triethylamine for 30 minutes. This reaction mixture is added drop-wise, at 0°C, to a solution of the above-described product **12** (18 mg in 0.2 ml DMF) and agitated for 3 hours at 0°C. The resulting product is precipitated by the addition of diethyl ether

and chromatographically purified as in example 5. The result is 12 mg (47%) of product 13.

## Patent Claims

1. Compounds of the general formula I

$$(F\text{-}L)_m\text{-}A \qquad (I)$$

where

F represents a dye molecule having at least one absorption maximum between 600 and 1200 nm,

L represents a linker structure which contains an acid-labile and/or enzymatically divisible bond,

m is a number between 1 and 80,

in which case, if m is a number between 1 and 3,

A represents a dye molecule with at least one absorption maximum between 600 and 1200 nm, an antibiotically or anticytostatically effective molecule, a biomolecule, a nonbiological macromolecule or a combination of $B\text{-}(L\text{-}W)_o$ or $D\text{-}(L\text{-}W)_o$, where

D is a nonbiological macromolecule,

B is a biomolecule,

L is as indicated above,

W represents an antibiotically or anticytostatically effective molecule,

o is a number between 1 and 20,

whereas, if m is a number between 4 and 80,

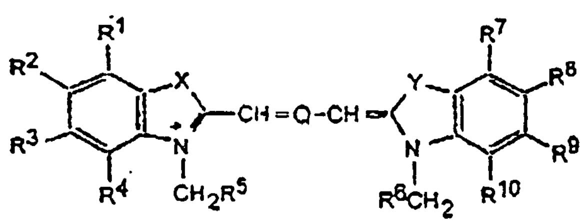
A represents a biomolecule, a nonbiological macromolecule or a combination of $B-(L-W)_o$ or $D-(L-W)_o$, where

D, B, L, W and o are as indicated above.

2. Compounds as in claim 1, **characterized in that**, in the general formula (I), F and/or A represent a polymethine dye, a tetrapyrrole dye, a tetraazapyrrole dye, a xanthine dye, a phenoxazine dye or a phenothiazine dye.

3. Compounds as in at least one of the preceding claims, characterized in that, in the general formula (I), P and/or A represent a cyanine, squarilium, croconium, merocyanine or oxonol dye.

4. Compounds as in at least one of the preceding claims, characterized in that, in the general formula (I), F and/or A represent a cyanine dye along the general formula (II)




(II)

where

$R^1$ to $R^4$ and $R^7$ to $R^{10}$ independently of one another represent a fluoro-, chloro-, bromo, -iodo atom or a nitro group or a residual $-COOE^1$, $-CONE^1E^2$, $-NHCOE^1$, $-NHCONHE^1$, $-NE^1E^2$, $-OE^1$, $-OSO_2E^1$, $-SO_2E^1$, $-SO_2NHE^1$, $-E^1$,

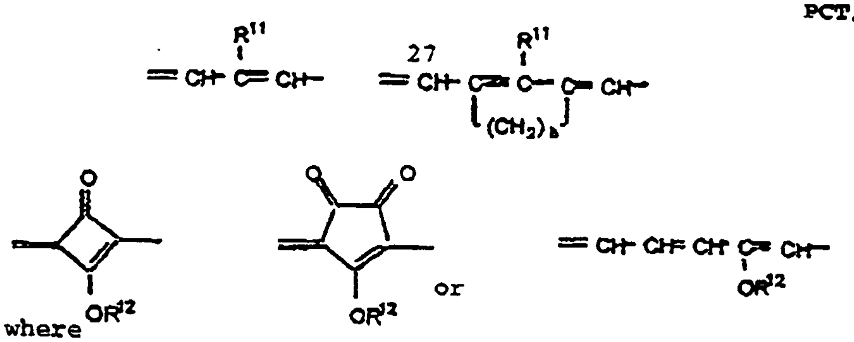
where $E^1$ and $E^2$ independently of one another represent a hydrogen atom, a saturated or unsaturated, branched or linear $C_1$-$C_{50}$-alkyl chain, where the chain or parts of the chain may under certain circumstances form one or several aromatic or saturated cyclic $C_5$-$C_6$ or bicyclic $C_{10}$ units and where the $C_1$-$C_{50}$ alkyl chain is interrupted by 0 to 15 oxygen atoms and/or 0 to 3 carbonyl groups and/or substituted with 0 to 5 hydroxyl groups, 0 to 5 ester groups, 0 to 3 carboxylic groups or 0 to 3 amino groups,

and where respectively adjoining residues $R_1$ - $R_4$ and/or $R_7$ - $R_{10}$, under formation of a six-link aromatic carbon ring, may be linked with one another,

$R^5$ and $R^6$ independently of each other represent a residue of $-E^1$ as indicated above or a $C_1$-$C_4$ sulfoalkyl chain,

and/or $R^1$ to $R^{10}$ represent a link with L,

Q is a fragment



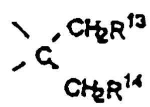
where

$R^{11}$ represents a hydrogen, fluoro, chloro, bromo, iodo atom or a nitro group or a residual $-NE^1E^2$, $-OE^1$ or $-E^1$ where $E^1$ and $E^2$ are as indicated above or represent a link with L,

$R^{12}$ represents a hydrogen atom or a residual $E^1$ per the above,

b is a number 0, 2 or 3,

X and Y independently of each other represent residual O, S, -CH-CH- or a fragment



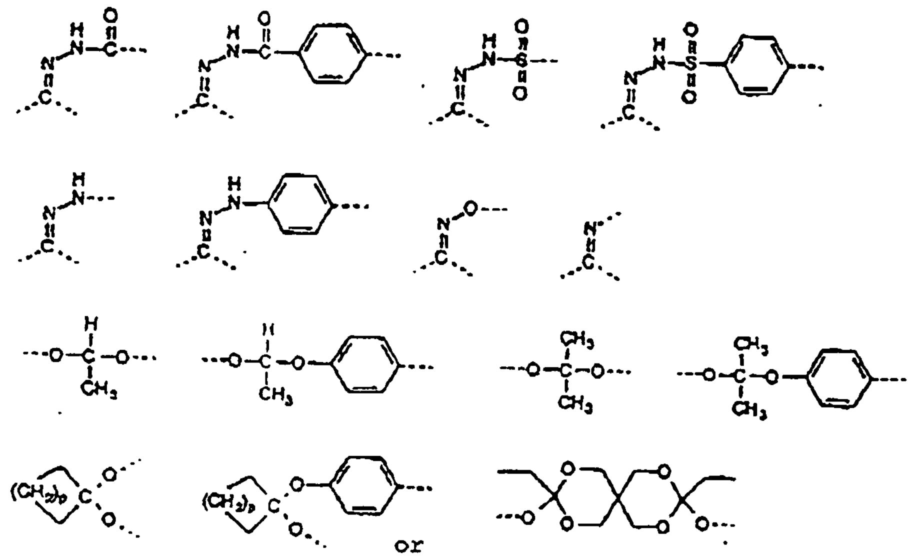
where

$R^{13}$ and $R^{14}$ independently of each other represent hydrogen, a saturated or unsaturated, branched or linear $C_1$ - $C_{10}$ alkyl chain which may be interrupted by up to 5 oxygen atoms and/or substituted with up to 5 hydroxyl groups and the residual $R^{13}$ and

$R^{14}$ may be linked with one another while forming a 5- or 6-link ring.

5. Compounds as in at least one of the preceding claims, characterized in that, in the general formula (I), W or A represent antibiotics, folic acid analogues, pyrimidine analogues, purine analogues, hormonally effective substances as well as other cytostatically effective substances.

6. Compounds as in at least one of the preceding claims, characterized in that, in the general formula (I), L represents a structure which contains an acid-labile fragment




where p is a number between 2 and 4.

7. Compounds as in at least one of the preceding claims, characterized in that, in the general formula (I), L represents a structure which

contains an enzymatically divisible chemical bond.

8. Compounds as in at least one of the preceding claims, characterized in that, in the general formula (I), L represents a structure which is split by cathepsines, peptidases, carboxypeptidases, α- and ß-glycosidases, lipases, phospholipases, phosphatases, phosphodiesterases, proteases, elastases, sulfatases, reductases and bacterial enzymes.

9. Compounds as in at least one of the preceding claims, characterized in that, in the general formula (I),
A and/or B represents an antibody, conjugates and fragments thereof, specific peptides and proteins, receptors, enzymes, enzyme substrates, nucleotides, natural or synthetic ribonucleic acids or desoxyribonucleic acids or chemical modifications thereof such as aptamers or anti sense-oligonucleotides, lipoproteins, lectines, carbohydrates, mono-, di- or trisaccharides, linear or branched oligo- or polysaccharides or saccharide derivatives or a dextrane.

10. Compounds as in at least one of the preceding claims, characterized in that, in the general formula (I), D represents polyethylene glycol, polypropylene glycol, polylysine or polylysine dendrimeres or their derivatives.

11. Use of compounds per claim 1 for the in-vivo diagnosis of diseased tissue areas employing NIR radiation as well as for the therapeutic treatment of diseased tissue areas.

12. Optical diagnostic agent for the in-vivo diagnosis of diseased tissue areas employing NIR radiation, characterized in that it contains at least one of the compounds per claim 1 in conjunction with traditional adjuvants and/or carrier substances as well as diluents.